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Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state

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SUMMARY

Objective: Mesenchymal stem cells (MSCs) are a promising cell type for the repair of damaged cartilage in osteoarthritis (OA). However, OA synovial fluid and factors secreted by synovium impede chondrogenic differentiation of MSCs, and the mechanism responsible for this effect remains unclear. In this study, we sought to investigate whether M1 and M2 synovial macrophages can contribute to the inhibition of MSC chondrogenesis.

Design: The constitution of synovial macrophage subsets was analysed by immunohistochemical staining of human OA synovium sections for CD86 (M1 marker) and CD206 (M2 marker). To assess the effect of synovial macrophages on chondrogenesis, collagen type II (*COL2*) and aggrecan (*ACAN*) gene expression were compared between MSCs undergoing chondrogenic differentiation in medium conditioned (CM) by human OA synovial explants, human synovial macrophages and fibroblasts, or peripheral blood derived primary human monocytes differentiated towards an M1 or M2 phenotype.

Results: OA synovium contained both M1 and M2 macrophages. Medium conditioned by synovial macrophages (CD45 + plastic adherent cells) down-regulated chondrogenic gene expression by MSCs. Additionally, CM of M1 polarised monocytes significantly decreased *COL2* and *ACAN* gene expression by MSCs; this effect was not observed for treatment with CM of M2 polarised monocytes.

Conclusion: MSC chondrogenesis is inhibited by OA synovium CM through factors secreted by synovial macrophages and our findings suggest that M1 polarised subsets are potential mediators of this anti-chondrogenic effect. Modulation of macrophage phenotype may serve as a beneficial strategy to maximise the potential of MSCs for efficient cartilage repair.

Introduction

The repair of damaged articular cartilage resulting from trauma or degenerative joint disease poses a major challenge due to the limited capacity of cartilage for self renewal, attributable to its avascular nature. Mesenchymal stem cells (MSCs) are considered a promising cell type for the repair of damaged cartilage due to their chondrogenic differentiation potential[1,2]. However, in order to achieve cartilage repair *in vivo*, chondrogenically differentiating MSCs are exposed to inflammatory mediators produced in response to injury or disease. Osteoarthritic synovial fluid and medium conditioned by synovium explants have been reported to inhibit the

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chondrogenic differentiation of MSCs[3,4]. Therefore the presence of a destructive inflammatory environment, as found in osteoarthritis (OA), may impede the use of MSCs in cartilage repair strategies.

The synovial membrane is an area of high functional importance within the joint, responsible for the production of synovial fluid which lubricates and nourishes chondrocytes. The membrane is composed of two cell types, synovial macrophages and fibroblasts. Synovial hyperplasia, increased vascular density and inflammatory cell infiltration are common features of OA[5]. Pro-inflammatory factors from synovial fluid of injured and osteoarthritic joints stimulate cartilage degradation and inhibit matrix synthesis[6–9]. Tumour necrosis factor-α (TNF-α), Oncostatin M (OSM), Interleukin (IL)-1β and IL-6 have been identified as some of the key players involved in synovial inflammation, with synovial macrophages considered to play a prominent role in the production of these mediators[10–13].

Macrophages exhibit a high degree of plasticity, having the potential to change phenotype according to environmental cues. They can be categorised as classically activated (M1), representing pro-inflammatory phenotypes, or alternatively activated (M2) macrophages, encompassing wound healing and regulatory subsets with anti-inflammatory properties[14]. M1 macrophages are associated with high production of pro-inflammatory mediators such as TNF-α, IL-6 and IL-12, and cell surface expression of the co-stimulatory molecule CD86, required for T cell activation and cytokine production[15,16]. Macrophages of the M2 subtype produce immunoregulatory factors including IL-10 and chemokine (C–C motif) ligand 18 (CCL18), and are associated with the surface expression of the mannose receptor (CD206)[17,18]. The M1-associated cytokines IL-6, IL1β and TNF-α induce destructive processes in chondrocytes including down regulation of collagen type II and aggrecan synthesis, as well as up regulation of matrix metalloproteinase-9 and cyclooxygenase-2 expression[19–23].

We hypothesise that M1 polarised macrophages mediate the earlier shown anti-chondrogenic effects of OA synovium on MSC chondrogenesis[3]. Our findings highlight a role of synovial macrophages in inhibiting the chondrogenic differentiation of MSCs. Furthermore, we identify M1 polarised subsets as key mediators of this anti-chondrogenic effect.

Methods

Synovium conditioned medium (SCM) preparation

OA synovial tissue was isolated from six patients (four males, two females; 63.2 ± 4.2 years) with advanced clinical OA, undergoing total knee replacement at Erasmus MC, University Medical Center, Rotterdam. OA was determined based on clinical evaluation of pain and radiographic changes, including narrowing of the joint space and osteophyte formation. Ethical approval was granted by the local ethical committee (MEC2004-322). Synovial tissue was cut in to pieces of 1–3 mm^2 and 200 mg of tissue was cultured in 1 ml of medium composed of Dulbecco's Modified Eagle's Medium (DMEM), GlutaMAX™ (Invitrogen, Carlsbad, CA, USA) supplemented with 1.5 μg/ml fungizone (Gibco, Carlsbad, CA, USA), 50 μg/ml gentamycin (Gibco), 1% insulin-transferrin-selenium (BD Biosciences, Erembodegem, Belgium), 25 μg/ml ascorbic acid-2-phosphate (Sigma–Aldrich, St. Louis, MO, USA) 40 μg/ml L-Proline (Sigma–Aldrich) and 1 mM sodium pyruvate (Gibco), referred to as basic culture medium (BCM). Culture medium was refreshed every 24 h, and SCM harvested at day 3 of culture.

Isolation of synovial macrophages and fibroblasts

OA synovial tissue from one patient (female, 59 years) was digested with collagenase B (Roche, Penzberg, Germany; 1 mg/ml) for 45 min at 37°C and gently minced through a 100 μm filter (Becton Dickinson, Franklin Lakes, NJ, USA). The resulting cell suspension was layered on top of 15 ml of Ficoll (Ficoll–Paque™ PLUS, GE Healthcare) and separated by density gradient centrifugation. The interphase layer was removed, washed in BCM and seeded at 500,000 cells/cm^2. Cells were incubated at 37°C for 15 min for a macrophage enriched population (MEP). Un-adherent cells were removed, transferred to a new well and incubated for 120 min to generate a fibroblast enriched population (FEP)[24,25]. MEP and FEP conditioned medium (CM) was harvested following 24 h of culture.

11-fibrau staining was assessed to confirm the enrichment of each fraction. 11-fibrau is specific for a 112-kDa molecule, localised to the cell surface of human fibroblasts[26–28]. Isolated synoviocytes cultured on glass slides were incubated at 37°C for 60 min for a MEP, and 12 h for a FEP fraction. Cells were fixed in 70% ethanol for 1 h, blocked with 10% goat serum (Sigma–Aldrich), and incubated for 1 h with monoclonal anti-human 11-fibrau (Imgen biosciences, MA, USA; 4.2 μg/ml). Slides were incubated for 30 min with a biotinylated anti-mouse link (Biogenex, fremount, CA, USA), diluted 1:50 with phosphate buffered saline (PBS)/1% BSA/5% human serum (CLB, The Netherlands), and thereafter with an alkaline phosphatise-conjugated streptavidin label (Biogenex) diluted 1:20 with PBS/1% BSA/5% human serum. Slides were incubated with Neu Fuchsin, $NaNO_2$, Naphtol AS-MX phosphate, Di-methylformamid with levamisole (all Sigma–Aldrich) in TRIS-HCl buffer (0.2 M, pH 8.5) in the dark for 30 min. Cells were counterstained with haematoxylin and slides mounted utilising VectaMount (Vectorlabs, Peterborough, UK).

To obtain synovial macrophages, synoviocytes were separated based on their expression of the haematopoietic cell marker CD45[29]. OA synovial tissue from sixpatients (one male, five females; 69.6 ± 7.5 years) was isolated and digested as previously described. Synoviocytes were incubated with anti-CD45-PE antibody (BD Biosciences; diluted 1:100) for 1 h in the dark at 4°C, and labelled with 30 μl of anti-mouse IgG magnetic bead solution (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min in the dark at 4°C. CD45 + populations were isolated by magnetic activated cell sorting (MACS, MACS Separation columns LS and MidiMACS™ Separator, Miltenyi Biotec). Purity of CD45 + populations was confirmed by flow cytometry, with average purity of 95.7% ± 3.1%. Purity of CD45 - populations was 66.5% ± 13.9%, therefore this condition was not used for subsequent experiments. CD45 + cells were seeded at 100,000 cells/cm^2 and cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Lonza, Verviers, Belgium) for 4 h. Cells were then washed to remove non-adherent populations and cultured in BCM for 24 h following which CM was harvested.

Human monocyte isolation and differentiation

Buffy coats were obtained from six healthy male blood donors (48.5 ± 19.3 years) at the Sanquin blood bank Rotterdam. Two buffy coats were used per experiment, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation[30]. PBMCs were labelled with 100 μl of anti-CD14 + magnetic bead solution (Miltenyi Biotec) for 20 min in the dark at 4°C and monocytes isolated by MACS.

Monocytes were seeded at 500,000 cells/cm^2 and cultured in X-Vivo™ 15 medium (Lonza) supplemented with 20% FBS, 50 μg/ml gentamycin and 1.5 μg/ml fungizone. Monocytes were stimulated with 10 ng/ml IFN-γ (PeproTech, Rocky Hill, NJ, USA) & 100 ng/ml lipopolysaccharide (LPS, Sigma–Aldrich) for 72 h for differentiation towards an M1 or 10 ng/ml IL-4 (PeproTech) for 72 h for an M2 phenotype. Cells were washed and cultured for 24 h in BCM, following which M1 or M2 CM was harvested.

Cytokine assays

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentration of IL-6 and CCL18 in SCM, M1 and M2 CM as per manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

Isolation and culture of human MSCs

MSCs were isolated from heparinised bone marrow aspirates taken from the iliac crest of a healthy volunteer (male, 35 years) and a patient with OA undergoing total hip replacement surgery (female, 77 years) with informed consent. All procedures for collection of bone marrow have been approved by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland (reference:2/08), the institutional National University of Ireland Galway Research Ethics Committee (reference:08/May/14) and local ethical committee of the Erasmus MC, University Medical Center Rotterdam (MEC2004-142). MSCs were isolated based on their plastic adherence and expanded in medium consisting of Minimum Essential Medium alpha (Invitrogen) supplemented with 10% FBS, 1 ng/ml fibroblast growth factor-2 (R&D systems), 25 μg/ml ascorbic acid-2-phosphate, 1.5 μg/ml fungizone and 50 μg/ml gentamicin.

For chondrogenic differentiation, MSCs (passage 3) were encapsulated in 1.2% low viscosity alginate powder (CP Kelco, Atlanta, GA, USA) dissolved in physiological saline at a density of 4×10^6 cells/ml. MSC alginate beads were formed by purging through a 23 gauge needle allowing droplets to fall into 102 mM $CaCl_2$ solution. Negative control MSC beads were cultured in incomplete chondrogenic medium (ICM) composed of BCM to which 0.1 μM Dexamethasone (Sigma–Aldrich) was added. For induction of chondrogenesis, MSC beads were cultured in complete chondrogenic medium (CCM), composed of ICM with the addition of 10 ng/ml transforming growth factor (TGF)-β1 (R&D systems). MSC beads were cultured for 14 days, following which medium was supplemented with CM from all conditions. 5% SCM, 10% FEP and MEP CM were added to MSC beads, which were previously identified as optimal concentrations. To allow for high variability in cell number between CD45 + cell preparations, the lowest cell number used to generate CM (0.15 μg DNA) was equivalent to a concentration of 50% CM, and all conditions were normalised to this concentration. To account for high variability in cell number between M1 and M2 differentiated monocytes, the average cell number used to generate M1 and M2 CM across all donors, was equivalent to a concentration of 20% CM (Table I). The effect of SCM ($n = 6$), FEP and MEP ($n = 1$), CD45+ ($n = 6$), M1 and M2 ($n = 3$) CM on chondrogenic gene expression, was assessed in experimental triplicate. Culture was continued for a further 3 days after which mRNA was isolated, or 14 days after which samples were harvested for DNA and glycosaminoglycan (GAG) quantifications.

Table I
Calculation of the percentage of M1 and M2 CM, based on macrophage cell number (μg DNA)

Donor	Condition	DNA (μg)	% CM
1	M1	1.64	19.95
	M2	1.12	29.28
2	M1	1.58	20.65
	M2	0.79	41.43
3	M1	3.00	10.88
	M2	1.67	19.51

The average cell number used to generate M1 and M2 CM across all donors was equivalent to a concentration of 20% CM.

Quantification of DNA content

MSCs were released from alginate after 28 days of culture by incubation with 55 mM sodium citrate in 20 mM ethylenediaminetetraacetic acid (EDTA), and digested overnight at 60°C with an equal volume of papain solution (250 μg/ml papain in 50 mM EDTA and 5 mM L-cystein (Sigma–Aldrich)). Macrophages were lysed with 500 μl TritonX-100 (Sigma–Aldrich) and sonicated on ice. The DNA content of all samples was determined following ribonuclease type 3 (12.5 μg/ml, Sigma–Aldrich) and heparin (415U/ml, Leo Pharmaceuticals, Ballerup, Denmark) treatment, utilising ethidium bromide (5 μg/ml, Gibco). Deoxyribonucleic acid sodium salt from calf thymus (Sigma–Aldrich) was used as a standard. DNA was quantified by spectrophotometric detection of ethidium bromide binding at 340 and 590 nm.

GAG measurement

GAG content of digested MSC alginate samples was determined spectrophotometrically at 530 and 590 nm after reaction with dimethylmethylene blue (Polysciences, Valley Road, Warrington, PA, USA); the pH was lowered to 1.75 for measurements in alginate samples using chondroitin sulphate from shark cartilage as the standard (Sigma–Aldrich)[31,32]. The effect of SCM ($n = 2$) on GAG content of MSCs was assayed in experimental triplicates.

Gene expression analyses of MSCs and macrophages

MSC alginate beads were dissolved following incubation with 55 mM sodium citrate at 4°C. The cell solution was centrifuged at 400*g* for 8 min, pellet resuspended in 1 ml RNAbee (TelTest, Friendswood, TX, USA) per 1×10^6 nuclei and stored at −80°C. 350 μl/well of buffer RLT (Qiagen, Hilden, Germany) was used for direct cell lysis of macrophages. RNA isolation was performed using a RNeasy Microkit (Qiagen) and nucleic acid content determined spectrophotometrically using a NanoDrop ND1000 spectrophotometer (Isogen Life Science B.V., The Netherlands) at 260 and 280 nm. cDNA synthesis was performed using RevertAid First Strand cDNA synthesis Kit (MBI Fermentas, Germany) according to manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) was performed in 20 μL reactions on cDNA using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) utilising Taqman Universal PCR mastermix (Applied Biosystems, Foster City, CA, USA) or SybrGreen (Eurogentec, Seraing, Belgium). Gene expression of *IL-6* was assessed as a marker of M1 and *CCL18* and macrophage mannose receptor 1 (*MRC1*) as markers of M2 macrophages[18,33–35]. Gene expression was assessed in differentiated macrophages ($n = 3$) in experimental triplicate. For MSCs, expression of the cartilage-related genes collagen type II (*COL2*) and aggrecan (*ACAN*) were assessed. Primer pairs were used to determine transcript levels of genes of interest using settings previously described[30,36,37]. Gene expression levels were normalised to Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and relative expression calculated using the $2^{-\Delta CT}$ method ($2^{-(\text{CT gene of interest}-\text{CT internal control})}$)[38].

Evaluation of macrophage subtypes in synovium

5 μm sections were cut from paraffin embedded OA synovial tissue from 6 donors. Sections were stained for CD86 as a marker of M1, or CD206 as a marker of M2 macrophages[15,18,39]. Sections were deparaffinised, washed and heat-mediated antigen retrieval performed. Following blocking with 10% goat serum, sections were incubated for 1 h with primary antibodies CD206 (Abcam, Cambridge UK; 2.5 mg/ml) or CD86 (Genetex, Irvine, CA, USA; 0.45 μg/

ml). Sections were incubated for 30 min with a biotinylated anti-rabbit Ig link diluted 1:50 with PBS/1%BSA, and thereafter with an alkaline phosphatase-conjugated streptavidin label diluted 1:50 in PBS/1%BSA. Colour development and processing of sections was performed as previously described.

Statistical analysis

Normality was verified using Kolmogorov–Smirnov and Shapiro–Wilk normality tests using SPSS 15.0. Student's *t*-test was used to analyse paired data. For paired data that was not normally distributed, Kruskal–Wallis test was performed, followed by the Mann–Whitney *U* test. The false discovery rate was calculated for each comparison to apply multiple testing correction. For all analyses, differences were considered statistically significant at $P < 0.05$.

Results

SCM inhibits MSC chondrogenesis

MSC alginate beads successfully underwent chondrogenesis in our differentiation system, as indicated by significantly increased *COL2* and *ACAN* gene expression in TGF-β only treated MSC alginate beads compared to untreated [Fig. 1(A)]. Gene expression levels of *COL2* were significantly lower ($P = 0.002$; average decrease of 75.2% ± 17.3%) in MSCs following treatment with 5% SCM, compared to MSCs not exposed to SCM [Fig. 1(B)]; this inhibitory effect was observed following treatment with SCM from all six donors, ranging from 44.2% to 95.2%. SCM from four of the six donors resulted in decreased expression levels of *ACAN* (ranging from 49.6% to 75.5%) in SCM-treated beads compared to untreated ($P = 0.27$; 41.9% ± 33.6% average decrease) [Fig. 1(B)]. Furthermore, SCM treatment significantly decreased ($P = 0.005$) GAG content of MSCs that were cultured for 28 days [Fig. 1(C)].

Additionally, the levels of IL-6 and CCL18 in SCM were quantified to evaluate the inflammatory state of synovium from each donor. All donors secreted high levels of IL-6 and lower levels of CCL18, albeit with high variability between donors [Fig. 1(D)].

Synovial macrophages are orchestrators of the inhibitory effect of osteoarthritic synovium on MSCs

To investigate whether synovial macrophages play a role in the detrimental effect of SCM on MSC chondrogenesis, isolated synoviocytes were separated to generate fibroblast-enriched (FEP) and macrophage-enriched (MEP) fractions based on the rapid adhesion of macrophages to tissue culture plastic. Morphological differences between cells in both fractions were clear. The MEP fraction displayed more cells with a rounded body which was associated with numerous filopodia, characteristic of macrophage morphology[40]. The FEP fraction contained predominantly elongated cells with cytoplasmic processes varying in shape and length [Fig. 2(A)]. Immunohistochemical stainings for the fibroblast membrane marker 11-Fibrau further confirmed cell enrichment of each fraction [Fig. 2(B)][28]. *COL2* gene expression levels in MSCs were reduced following treatment with 10% MEP CM. Treatment with 10% FEP CM however, did not negatively impact *COL2* expression [Fig. 2(C)] suggesting that synovial macrophages may be responsible for the anti-chondrogenic effect of OA synovium. To further investigate this observation, synoviocytes were isolated from six synovium donors and separated based on their expression of the haematopoietic cell marker CD45[29]. CM from CD45 + populations reduced *COL2* and significantly decreased *ACAN* gene expression levels ($P = 0.01$) in MSCs [Fig. 2(D)].

M1 and M2 macrophages are present in end-stage OA synovium

Having identified a role for synovial macrophages in the inhibition of chondrogenic gene expression in MSCs, we sought to

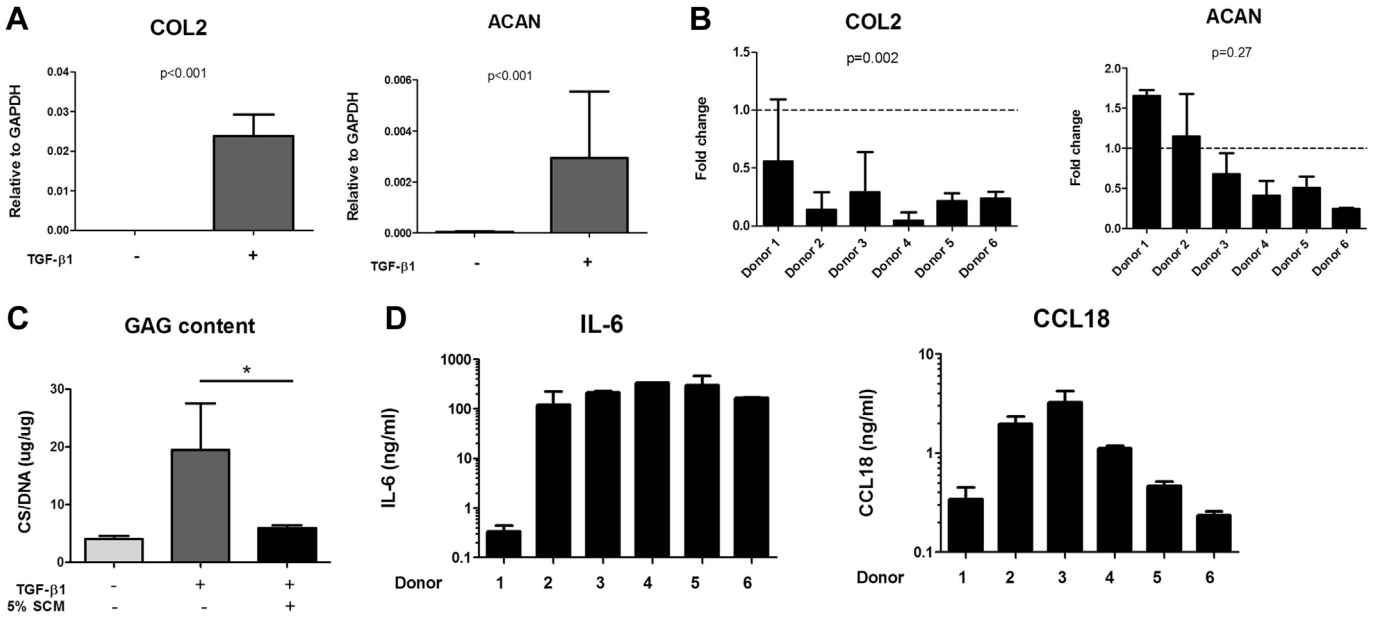

Fig. 1. Osteoarthritic SCM negatively affects chondrogenic differentiation of MSCs. MSC alginate beads were treated with 5% SCM generated from six OA synovium donors at day 14 of chondrogenesis. (A) TGF-β stimulation of MSC alginate beads significantly increases *COL2* and *ACAN* gene expression ($P < 0.001$). Values represent the mean ± SD of experimental triplicates with one MSC donor. (B) Fold change in *COL2* and *ACAN* gene expression levels compared to TGF-β stimulated only control following 3 days of 5% SCM treatment. Values represent the mean ± SD of experimental triplicates. Statistical significance was determined by Kruskal–Wallis test followed by Mann–Whitney *U* test. The overall inhibitory effect on *COL2* was significant ($P = 0.002$). (C) GAG content of MSC alginate beads at day 28 of chondrogenesis, following 5% SCM treatment for 14 days. Values represent the mean ± SD of experimental triplicates with SCM from two synovium donors. Statistical significance was determined by Kruskal–Wallis test followed by Mann–Whitney *U* test. * = $P < 0.05$. (D) ELISA analysis and quantification of IL-6 and CCL18 levels in SCM of six donors with OA.

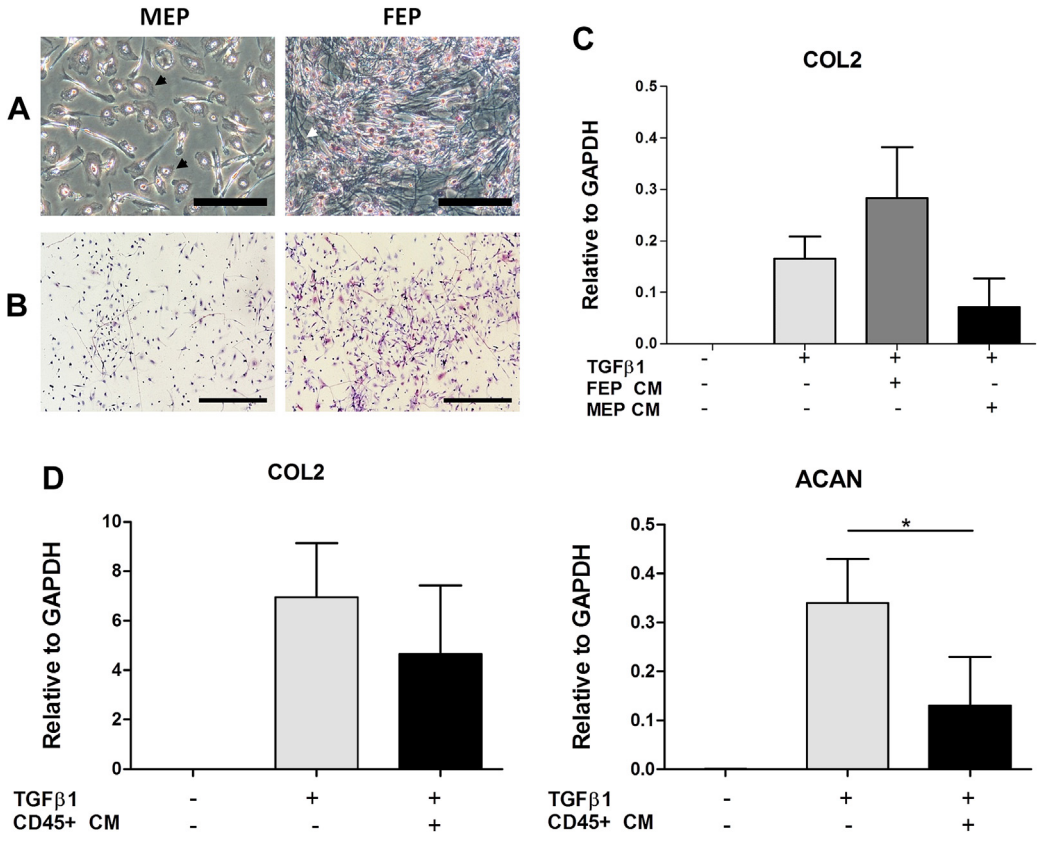

Fig. 2. Synovial macrophages inhibit chondrogenic gene expression in MSCs. MSC alginate beads were treated with 10% CM generated from synovial macrophage enriched (MEP) or fibroblast enriched (FEP) populations (one synovium donor), and 50% CD45 + population CM (six synovium donors). (A) Phase contrast images of Haematoxylin-eosin stained MEP and FEP. Black arrows indicate characteristic macrophage morphology, and white arrows indicate characteristic fibroblast morphology of both fractions. Scale bar indicates 50 μm. (B) Staining of MEP and FEP for the fibroblast marker 11-fibrau. Scale bar indicates 150 μm. (C) Inhibition of *COL2* gene expression in MSCs following stimulation with MEP CM. Values represent the mean ± SD of experimental triplicates and CM generated from one synovium donor. (D) Inhibition of *COL2* and *ACAN* gene expression in MSCs following stimulation with 50% CD45 + population CM. Values represent the mean ± SD of six synovium donors, each assessed in experimental triplicate. Statistical significance was determined by Kruskal–Wallis test followed by Mann–Whitney *U* test. * = $P < 0.05$.

identify whether M1 or M2 macrophages could be responsible for this effect. Immunohistochemical analyses for CD86 as an M1 and CD206 as an M2 marker confirmed the presence of both subsets in OA synovium sections from six donors. However, large variation in the level of expression of each marker between donors was observed [Fig. 3]. Expression of the M1 marker CD86 was primarily localised to the synovial lining layer in two donors (donor 3&4), with dispersed expression throughout the sublining region. One donor (donor 6) contained CD86 expressing cells predominantly in the sublining layer. Conversely, three donors (donor 1, 2 & 5) did not express this marker. We detected expression of the M2 marker CD206 in four donors (donor 2, 3, 4 & 5), throughout the synovial

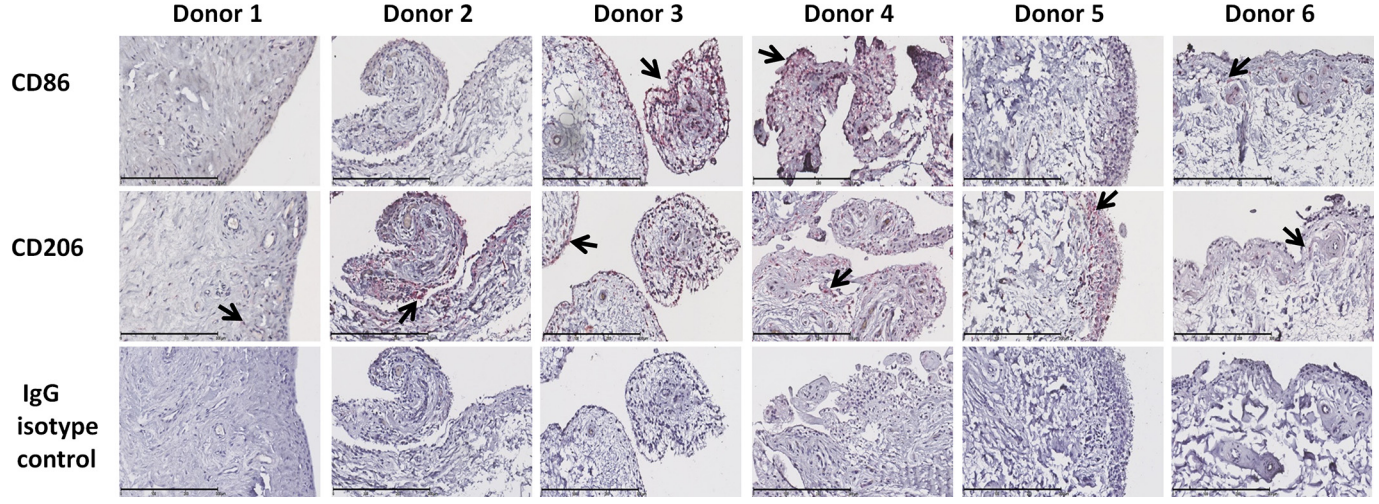

Fig. 3. M1 and M2 macrophages are present in OA synovium. Photomicrographs of OA synovium stained with anti-CD86, anti-CD206 or IgG isotype control antibody. Images are representatives of paired sections from six donors. Black arrows indicate positive cells, scale bare indicates 300 μm.

lining and sublining layers. Two donors contained few CD206 expressing cells localised to the sublining region (donor 1 & 6).

M1 polarised macrophages negatively impact MSC chondrogenesis

Medium conditioned by M1 differentiated monocytes contained significantly higher levels of the M1-associated cytokine IL-6 ($P = 0.039$), but low levels of the M2-associated chemokine CCL18. Conversely, medium conditioned by M2 differentiated monocytes did not contain IL-6, but CCL18 was significantly ($P = 0.0278$) increased [Fig. 4(B)]. Moreover, differentiated monocytes exhibited *IL6*, *CCL18* and *MRC1* gene expression profiles of M1 and M2 polarised cells [Fig. 4(A)]. This characteristic pattern of cytokine and gene expression was observed with each of the three biological replicates. Treatment of chondrogenic MSCs with 20% M1 CM significantly inhibited *COL2* ($P < 0.001$) and *ACAN* ($P < 0.001$) gene expression compared to untreated chondrogenic MSCs (80% ± 14.1% and 86.8% ± 11.5% decrease, respectively) [Fig. 4(C)]. This inhibitory effect was observed with M1 CM from each biological replicate (ranging from 63.8% to 89.7% for *COL2*, and 73.6% to 94.5% for *ACAN*). We did not observe any significant difference in *COL2* gene expression levels between MSCs treated with 20% M2 CM and untreated MSCs ($P = 0.124$). However, treatment with 20% M2 CM did significantly reduce *ACAN* gene expression ($P = 0.002$; average decrease of 27.3% ± 9.9%, ranging from 17.2% to 36.9%).

Discussion

The inhibitory effect of factors secreted by OA synovium on MSC chondrogenesis has been previously reported, however, the exact mechanism responsible for this effect has yet to be elucidated[3,4]. In this study, we report that synovial macrophages isolated from OA synovium orchestrate the negative impact of OA synovium on MSC chondrogenesis. Furthermore, we show that M1 polarised subsets

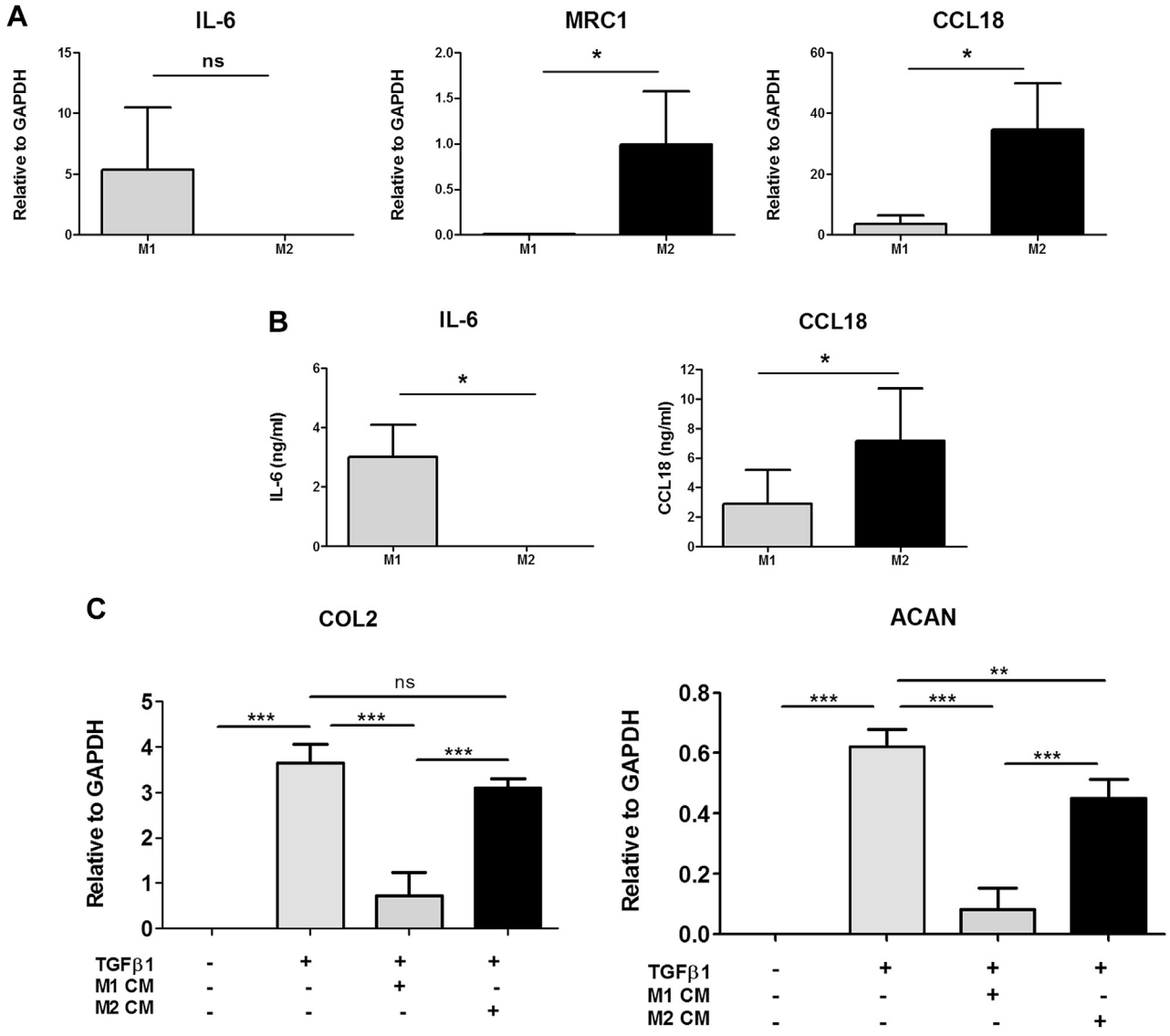

Fig. 4. M1, and not M2, macrophages inhibit chondrogenesis of MSCs. Primary monocytes (3 biological replicates) were differentiated towards an M1 or M2 phenotype following stimulation with IFN-γ & LPS or IL-4 for 3 days. (A) Gene expression of the M1 marker *IL-6* and the M2 markers *CCL18* and *MRC1* in M1 or M2 stimulated macrophages. Values represent the mean ± SD of three biological replicates, statistical significance was determined by a paired Student's *t*-test, * = $P < 0.05$. (B) ELISA analysis and quantification of IL-6 and CCL18 levels in M1 and M2 macrophages. Values represent the mean ± SD of three biological replicates, statistical significance was determined by a paired Student's *t*-test, * = $P < 0.05$. (C) Gene expression of *COL2* and *ACAN* following stimulation of MSC beads with 20% M1 or M2 CM for 3 days. Values represent the mean ± SD of macrophage biological triplicates, each assayed in experimental triplicate. Statistical significance was determined by a Kruskal–Wallis test followed by Mann–Whitney *U* test, ** = $P < 0.005$, *** = $P < 0.001$.

inhibit chondrogenic differentiation of MSCs. These findings may implicate M1 synovial macrophages as key regulators of the anti-chondrogenic effect of OA synovium.

Pro-inflammatory mediator production by inflamed synovium is a feature of OA pathology, postulated to drive destructive events in neighbouring chondrocytes[5,9]. Synovial macrophages play a key role in the production of such factors, however, little is known regarding the effect of macrophage subsets on MSC chondrogenesis. We confirmed the presence of different macrophage subsets by CD86 and CD206 immunohistochemical staining on OA synovium sections. In addition we have analysed secretion of an inflammatory cytokine IL6 and an anti-inflammatory cytokine CCL18. Remarkably, SCM from donor 1 contained less IL-6 and CCL18 than SCM from other donors, and failed to reduce *COL2* and *ACAN* gene expression in MSCs. These findings may suggest that the anti-chondrogenic effect of OA synovium is dependent on its inflammatory status. Interestingly, Gierman *et al.* have detected comparable expression levels of inflammatory mediators by end-stage OA and normal synovial tissue, and an effect of OA synovium on GAG release from cartilage explants was not observed[41]. Previous studies have demonstrated that OA synovium does not affect GAG release but inhibits GAG synthesis in cultured osteoarthritic cartilage explants[7]. We have observed an inhibitory effect of OA SCM on the expression of *ACAN* and *COL2* in MSCs during chondrogenic differentiation, and significantly reduced GAG content compared to MSCs not exposed to SCM. In this system, as in cartilage explants, end stage OA synovium may not directly induce cartilage degradation and GAG release, but adversely affect GAG production.

A mixed expression pattern of M1 and M2 macrophages was previously detected in the synovium of patients with OA, and macrophages localised to the synovial lining preferentially expressed markers of M2 polarised cells[42]. However, we detected similar levels of M1 and M2 markers localised to the lining layer in end-stage OA synovial tissue. M1 and M2 CM, prepared from human blood derived monocytes polarised to an M1 or M2 phenotype, were used as a model system to address the effect of both subsets on MSC chondrogenesis. The significant difference in protein and gene expression of IL6 and CCL18 between M1 and M2 cells, validate the use of M1 and M2 CM as a model system for macrophage subtypes. Corresponding with OA SCM, M1 CM significantly decreased *COL2* and *ACAN* gene expression of MSCs, where inhibition of *COL2* was not observed following treatment with M2 CM. Although M2 CM treatment inhibited *ACAN* gene expression, this inhibitory effect was less than the effect observed with M1 CM. Interestingly, M1 polarised macrophages have been reported to inhibit the proliferation and viability of MSCs *in vitro*; this effect was not observed with cells and mediators of the M2 subset[43]. However, we did not observe any difference in DNA content between M1 CM, M2 CM or untreated chondrogenic MSCs, suggesting that M1 polarised cells elicit an unfavourable effect in our system by directly impacting chondrogenic gene expression.

For proof of principle that synovial macrophages are responsible for the inhibitory effect of OA synovium, the effect of CM from synovial fibroblast and macrophage enriched populations on chondrogenesis was assessed. Our initial findings suggest that synovial macrophages are primary mediators of the anti-chondrogenic effect. Given the unrefined nature of this technique, we sought to isolate synovial macrophages based on their expression of CD45[29]. CD45 + CM negatively impacted chondrogenic gene expression by MSCs. Although we achieved 95.7% purity of CD45 + populations, purity of CD45- populations was 66.5% and therefore not utilised for our experiments. Given the technically challenging nature of this technique, further optimisation may be required to isolate pure populations and fully clarify the contribution of synovial fibroblasts to the inhibitory effect of OA synovium. Advanced OA synovium has been reported to contain CD45 + natural killer and T cell infiltrating populations, and we cannot rule out the presence of these populations in our system[44]. However, we believe that the removal of non-adherent cells prior to conditioning of medium may have depleted such leucocyte populations which do not adhere during culture[45].

An MSC alginate bead system was utilised to assess the chondrogenic differentiation of MSCs, and the suitability of this system has been previously reported[46]. Randeau *et al.* observed comparable levels of proteoglycan production and gene expression of *COL1*, *COL2* and *COL10* between chondrogenically differentiating MSCs cultured in alginate beads and pellets. In previous studies we have used pellet cultures to investigate the effect of SCM[47,48], and have confirmed that the effect on *COL2* and *ACAN* gene expression is similar in alginate and pellet cultures. Furthermore, MSCs in alginate have formed cartilage matrix *in vivo*[49,50]. Together, these findings validate the use of the alginate system for MSC chondrogenesis.

Our study has some limitations. Firstly, MSCs from one donor were used per experiment to analyse the effect of SCM, FEP, MEP, CD45+, M1 or M2 CM on chondrogenesis. However, each experiment demonstrated a clear inhibitory effect. Moreover, in previous experiments we observed a strong negative effect of OA-SCM, pooled from seven donors, on the chondrogenic differentiation of different MSC donors in pellet culture[47,48]. Given that the negative impact of SCM on MSC chondrogenesis is consistent, we believe that the use of one MSC donor per experiment is sufficient. A second limitation includes the restriction of our analyses to IL-6 and CCL18 as indicators of the inflammatory profile of synovium from each donor. Although IL-6 has been previously described to be abundantly present in OA SCM, and *in vitro* cell cultures have associated these cytokines with M1 and M2 macrophages respectively, caution should be applied when making any association between the presence of these factors in OA SCM and synovial macrophages[7]. Furthermore, this is an *in vitro* study using differentiated human monocytes to model the effect of synovial tissue resident macrophages on MSC chondrogenesis. From our data, we have observed an inhibitory effect of M1 differentiated macrophages, however further analysis is required to clearly elucidate a role of M1 polarised synovial tissue macrophages.

In summary, we have confirmed the inhibitory effect of OA SCM on the chondrogenic differentiation of MSCs and have identified M1 polarised macrophages as potential mediators of this anti-chondrogenic effect. We propose that modulation of synovial macrophage phenotype towards an anti-inflammatory state or attenuation of M1 mediators may be of vital importance for the development of efficient MSC-based cartilage regeneration strategies.

Contributions

NF and MdV-vM acquired, analysed and interpreted data, drafted and edited the article. GvO conceived this study and its design, interpreted the data, drafted and edited this article.

JL and WW acquired, analysed and interpreted data. NG provided technical support with data acquisition. YMB-J, EF, PvdK and JM interpreted data. All authors critically revised the article for important intellectual content and approved the final version to be submitted.

GvO, NF and MdV-vM take responsibility for the integrity of the work as a whole, from inception to finished article.

Competing interest statement
The authors declare no competing interests.

Role of the funding source
Study sponsors had no involvement with study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

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